The use of infrared spectroscopy as a test for species-specific pedal mucus in gastropod molluscs — a comparative study in Moreton Bay and Singapore

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ABSTRACT

Infrared (IR) spectroscopy was used to study pedal mucous characteristics of eight species of marine gastropods to determine if it was useful for species identification. Three species of *Nerita*, two morphologically indistinguishable species of *Littoraria* (*L. articulata* and *L. strigata*) and one species each of *Nodilittorina*, *Austrolittorina* and *Echinolittorina*, were compared with a terrestrial snail, *Achatina fulica*. The IR spectra all showed high absorption in the 900–2100 cm^{-1} wave number region. No benzene overtones were observed at low wave numbers (1800–2000 cm^{-1}) suggesting that aromatic amino acids could be lacking in core glycoprotein molecules. Strong absorption bands at peaks of 1644 cm^{-1} and 1545 cm^{-1} were obtained for all species and attributable to amide-I and amide-II peaks respectively. Amide I (range 1600–1720 cm^{-1}) to Amide II (1500–1600 cm^{-1}) band area ratios ranged significantly: *Nerita* species: 1: 0.10–1: 0.22; *Achatina fulica* 1: 0.40; and *Littoraria* sp. 1: 0.48; *Austrolittorina unifastciata*: 1: 0.57; *Echinolittorina malaccana*: 1: 0.65 and *Nodilittorina pyramidalis*: 1: 0.74. These differences appear useful for distinguishing between the six genera and congeneric species studied. ❑ *infrared, intertidal, snail, spectroscopy, Singapore, marine, Moreton Bay, mucus, Queensland.*

Molluscan mucus is made up of many compounds such as lectins, charged muco-polysaccharides, glycoproteins, proteins, uronic acid, sialic acid hexosamine and a host of other molecules in an aqeous medium (Schlichter 1982; Cottrell *et al.* 1993, 1994; Furuta *et al.* 1995; Davies & Hawkins 1998). It is generally used for the protection of cell surfaces exposed to the external environment (Davies & Hawkins 1998). Information about the mollusc's sexual state and direction of locomotion is also conveyed to conspecifics and predators by the mucus (Denny 1989). The mud snail, *Ilynassa obsoleta*, (Say, 1822) follows polarised trails of conspecifics in the pursuit of mates, but ignores non-conspecific trails (Bretz & Dimock 1983). Similarly, Erlandsson & Kostylev (1995) reported that male *Littorina littorea* track females using cues from conspecific trails, but ignore the trails of other males. Thus, both species-specific and gender specific cues are known to occur within gastropod mucus.

Cottrell *et al.* (1993) used SDS-PAGE electrophoresis of mucus to distinguish between seven snail species, however, such analysis requires complicated preparation and purification of the sample. Recently, infrared (IR) spectroscopy has been proposed as a simple method to study molluscan mucus (White *et al.* 1997; Skingsley *et al.* 2000), as lengthy sample preparation is unnecessary and results of spectra analyses can be obtained within seconds. Skingsley *et al.* (2000) successfully used IR spectroscopy of mucus to distinguish between six species of slugs, using two species of terrestrial snails, *Helix aspersa* and *Cepaea nemoralis* for comparison.

The identification of many mollusc species is difficult, and often requires dissection to study subtle differences in internal organ structure. Reid (1986) reported that it was difficult to distinguish between *Littoraria strigata* and *L. articulata* merely by their shells, and additional examination of anatomical features (e.g. penial form) was required. Lee (2003) when conducting a field study on the distribution, modes of attachment, and heat tolerance of Singapore littorinids, had to lump together *Littoraria articulata* and *Littoraria strigata* under *Littoraria* sp., as the shells of these two species are too similar in colour and morphology to be able to separate in the field. The objective of the present study was to test IR spectroscopy of pedal mucus for its efficacy in distinguishing between eight species in the genera, *Austrolittorina*, *Echinolittorina*, *Nodilittorina*, *Nerita* and *Littoraria*.

MATERIALS AND METHODS

Five individuals of *Austrolittorina unifasciata* (Gray, 1826) (= *Nodilittorina unifasciata*, see Reid, 2007) and *Nodilittorina pyramidalis* (Quoy & Gaimard, 1833) were collected from the high intertidal rocky shore at Hospital Bay, Dunwich, North Stradbroke Island on 23 February 2005, and kept in moist containers for transport back to the laboratory in Singapore. Similarly several individuals of *Echinolittorina malaccana* (Gray, 1839) (= *Nodilittorina trochoides*, see Reid, 2007), *Nerita lineata* Gmelin, 1791, *Nerita planospira* Anton, 1839, *Nerita chamaeleon* Linnaeus, 1758, and *Littoraria* sp. were also taken from the Pasir Ris Nature Area in Singapore. Five individuals of the giant African snail, *Achatina fulica* Bowdich, 1822 were collected from Singapore. The spectrum obtained from the pedal mucus of this terrestrial pulmonate was used to compare with the marine gastropods' spectra. Specimens were used within five days of collection. Marine gastropods were wetted with artificial seawater to induce them to crawl, while tap water was used for the giant African snail. Snails may produce a defense mucus that could have properties different from normal body mucous secretions, and therefore the snails were handled several times in order to habituate them. As long as the stimulus is non-threatening, habituation is rapid (Carew & Sahley 1986).

The entire foot of each snail was swiped across a barium fluoride crystal IR window, thereby coating it with a thin layer of mucus. This mucus was analysed 'raw' which reduces the chance of artifact from any derivative procedure. Infrared spectroscopy in transmission mode was used, i.e., a spectroscopic beam was passed through the sample and crystal. The mucus was too viscous for injection into a normal transmission cell. The presence of liquid water, water vapour and barium fluoride bands disrupt the spectral profile, and therefore these were subtracted off-line from the raw mucus specimen. The corrected spectra were assigned bands that equate to the likely chemical components of the mucus using standard banding protocols developed by Williams & Fleming (1987). The IR spectra were recorded at a resolution of 2 cm^{-1} in the spectral range of 900–2000 cm^{-1} using a Perkin-Elmer Fourier transform infrared spectrometer. The sampling procedure was repeated five times for each species, and several individuals were used to reduce the likelihood of biological variability affecting the results.

RESULTS

The IR spectra of all gastropods show high absorption in the 900–2000 cm^{-1} wave number region, and this region contains particularly significant information on the organic components of the mucus (see Williams & Fleming 1987). In all species studied, there were strong absorption bands at peaks of 1644 cm^{-1} and 1545 cm^{-1}. These bands show the presence of amide bonds in the core proteins of both glycosaminoglycans (GAGS) and proteoglycan molecules, including lectins (Davies & Hawkins 1998), and are attributable to amide-I and amide-II peaks respectively. The shape of the amide bands, and the size of the area under them, provide useful information on the presence of β-sheets and β-turns (Surewicz *et al.* 1993).

The broad absorption band around the 1400 cm^{-1} region indicates the presence of different ionisation states of the carboxylate groups associated with acid derivatives of sugars and some amino acid side chains ($COOH \leftrightarrow COO^- + H^+$). Generally, CH_2 vibration occurs at 1450 cm^{-1} and CH_3 vibration at 1380 cm^{-1} and bands are due to the presence of the core carbon spines of

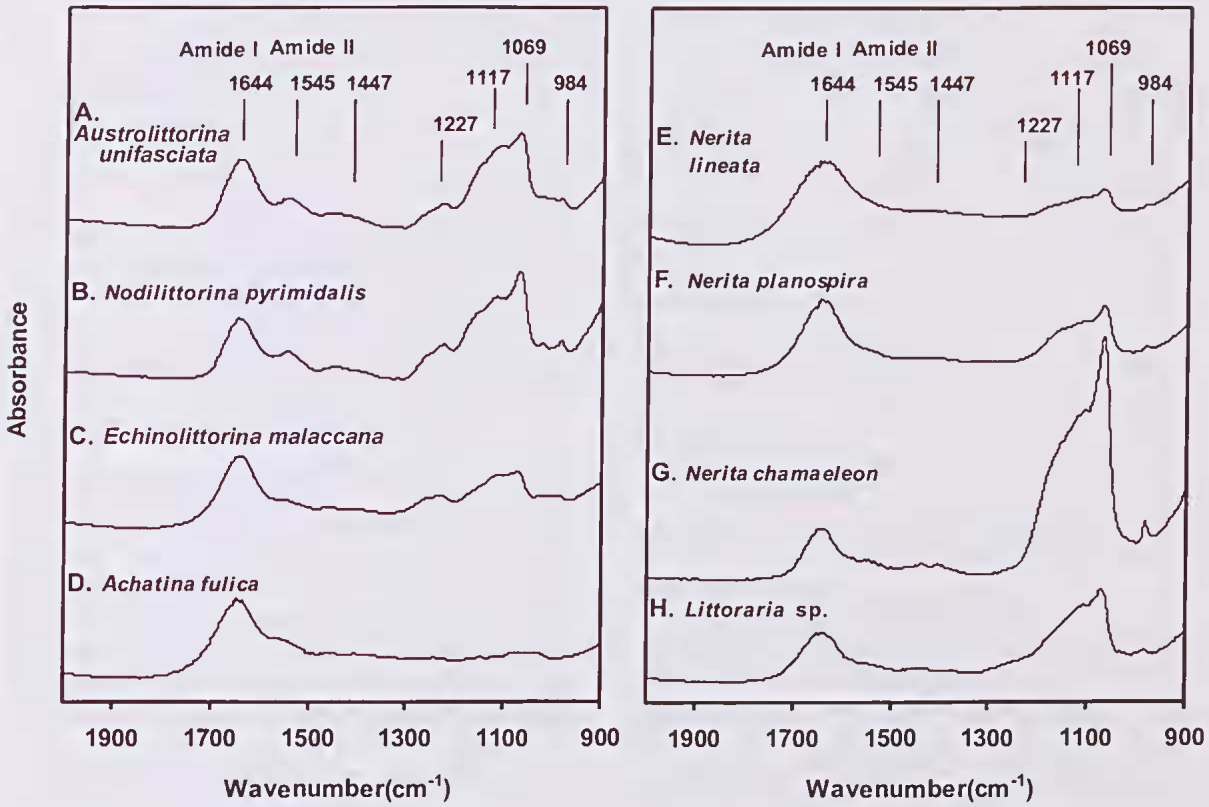

FIG. 1. Infrared spectra of the pedal mucus from: A, *Austrolittorina unifasciata*; B, *Nodilittorina pyramidalis*; C, *Echinolittorina malaccana*; D, *Achatina fulica*; E, *Nerita lineata*; F, *Nerita planospira*; G, *Nerita chamaeleon*; H, *Littoraria* species.

proteins, carbohydrates, and possibly lipids. A series of IR absorption peaks in the 950–1300 cm^{-1} region show the presence of a large quantity of OH and O-glycosidic bondings, as well as the occurrence of sulphated, acylated, and esterised compounds — all species in this study showed these peaks.

The typical IR spectra of *Austrolittorina unifasciata*, *N. pyramidalis* and *E. malaccana* mucus are shown in Fig. 1A–C. The measurements of band areas of Amide I and Amide II peaks at 1644 cm^{-1} and 1545 cm^{-1} respectively were obtained using software included in the Perkin-Elmer spectrometer system. These peaks indicate that protein amides with β-sheeting and β-turns are present in all three species. Table 1 shows the band area ratios of Amide I (1600–1720 cm^{-1}) to Amide II (1500–1600 cm^{-1}) for these three littorinid species. The band area ratios were determined with an accuracy of 0.01. The differences in these ratios reflect relative amounts of molecular ionisation and hydration. The band area ratio of *E. malaccana* (from Singapore) lies between that of the two Australian littorinid species.

Figure 1D shows the IR spectrum of the terrestrial snail, *Achatina fulica*. Absorption peaks at 1644 cm^{-1} and 1545 cm^{-1} indicate the presence of Amide I and Amide II respectively, but the band area ratio of 1:0.40 is smaller than that of all three littorinid species, and appears to reflect significant generic and specific level separation (Table 1).

The IR spectra of *Nerita lineata*, *N. planospira*, and *N. chamaeleon* show a strong Amide I peak at 1644 cm^{-1}, but a very weak Amide II peak at 1545 cm^{-1} (Fig. 1E–G), and consequently the band area ratios are much lower than those of all three littorinid species and of *Achatina fulica* (Table 1). Thus the relative amounts of molecular ionisation and hydration in the mucus of

Table 1. Band area ratios of Amide I (at 1644 cm^{-1}) to Amide II (at 1545 cm^{-1}) in infrared spectra of pedal mucous from species of *Austrolittorina*, *Nodilittorina*, *Echinolittorina*, *Achatina*, *Nerita* and *Littoraria*.

Genus	Species	Locality	Band area ratios
Austrolittorina	*A. unifasciata*	Moreton Bay, Australia	1:0.57
Nodilittorina	*N. pyramidalis*	Moreton Bay, Australia	1:0.74
Echinolittorina	*E. malaccana*	Pasir Ris Nature Area, Singapore	1:0.65
Achatina	*A. fulica*	National Institute of Education, Singapore	1:0.40
Nerita	*N. lineata*	Pasir Ris Nature Area, Singapore	1:0.10
	N. planospira	Pasir Ris Nature Area, Singapore	1:0.15
	N. chamaeleon	Pasir Ris Nature Area, Singapore	1:0.22
Littoraria	*L. articulata* +*L. strigata*	Pasir Ris Nature Area, Singapore	1:0.48

the *Nerita* species are significantly different from those of the other species in this study. The band area ratio for the samples of the cryptic *Littorina* species-pair (*L. articulata* and *L. strigata*) was 1: 0.48. This value was quite consistent between individuals and no significant differences were detected that might help reflect species differences. The band area ratio for *Nerita* is intermediate between that of the terrestrial genus *Achatina* (*A. fulica*) and those of the intertidal littorinid species in this study (Table 1).

DISCUSSION

The different Amide band ratios indicate that protein cores of the mucus of the individual species are different although all showed the presence of β-sheet and β-turn structural elements. The Amide band area ratios formed distinct groupings consistent with the six genera studied (Table 1) and thus may have generic significance although a greater range of species within each genus will need to be analysed before this can be conclusively stated. Different values for band area ratios were obtained for each species (Table 1), hence there is potential for reliable congeneric specific separation. With an experimental accuracy of 0.01, the band area ratio values (Table 1) are distinct for each species, i.e., the ratio values do not overlap and species-specific differentiation is possible.

In contrast, Skingsley *et al.* (2000) reported that there was little difference in the band area ratios for all the gastropods in their study, and only provided the ratio for *Arion ater* var. *rufus* (Linnaeus, 1758), i.e., 1: 0.68. This ratio of 1: 0.68, though close to *E. malaccana*'s 1: 0.65 and *N. pyramidalis*' 1: 0.74 values, is distinct from all ratio values reported in the present study. This suggests the possibility of species differentiation using band area ratios. Unfortunately individual band area ratios for the other species studied by Skingsley *et al.* (2000), i.e., *Arion subfuscus* (Draparnaud, 1805), *Arion hortensis* Férussac, 1819, *Deroceras reticulatum* (Muller, 1774), *Deroceras carnanae* (Pollonera, 1891), *Limax maculatus* (Kaleniczenko, 1851), *Helix aspersa* Muller, 1774, and *Cepaea nemoralis* Linnaeus, 1758, were not reported. More IR spectroscopy analyses of the mucus of other mollusc genera are needed.

Similar absorption peaks in the 900–1450 cm^{-1} wave number region were observed for all three rough periwinkle species as shown in the IR spectra (Fig. 1A–C). The 1447 and 1227 cm^{-1} peaks indicate the presence of carbon spin of the core proteins in the form of CH_2 and CH_3 relating to the composition and ionisation state of the amino acid side chains and sugar side chains (e.g., COOH, COO^-). In this spectral region, the absorption peaks at 984, 1069, 1117 cm^{-1}, and some weaker peaks which overlap with the broad bands around 1450 cm^{-1} region are all indicative of sulphated, acylated and esterified molecular structures in the mucus. In this regard our three littorinid species are very similar to the aquatic species *Lymnaea stagnalis* (Linnaeus, 1758), studied by Skingsley *et al.* (2000), and all differ from the terrestrial species *Arion subfuscus*, *Helix aspersa*, and *Arion ater* var. *rufus*

studied by Skingsley *et al.* (2000), because of the absence of a 1385 cm^{-1} peak in the IR spectra that is present in those species.

The IR spectra of all three *Nerita* species show similar features in the 900–1450 cm^{-1} region (Fig. 1E–G). Peaks at 984, 1069 and 1117 cm^{-1} were clearly observed. However, in this region, the spectra of the *Nerita* species lack the 1227 cm^{-1} peak present in the three littorinid genera. This IR peak can probably be used to differentiate the two genera. Furthermore, the IR features in the 900–1450 cm^{-1} region for the marine *Nerita* species (Fig. 1E–G) differ significantly from that of the terrestial *Achatina fulica* (Fig. 1D).

The IR spectrum of *Achatina fulica* shows the peaks in the 900–1450 cm^{-1} region are much weaker and different from those in the three species of the genus, *Nerita* as well as from that of the other three genera, *Austrolittorina*, *Echinolittorina* and *Nodilittorina* (Fig. 1D). The weak absorption peaks at 1033, 1070, and 1150 cm^{-1} for *Achatina fulica* were absent in the spectra of the three littorinid species. The differences in the IR features clearly indicate differences in the composition and ionisation state of the amino acid side chains and sugar side chains (e.g., COOH, COO^{-}), as well in the level of sulphated, acylated and esterified structures in the species. These differences are sufficient to set the pulmonate *Achatina fulica* apart from all the intertidal species in the present study.

Cottrell *et al.* (1993) reported that the aromatic amino acids in the core of glycoprotein molecules are represented by benzene ring overtones in the low wave number region from 1800–2000 cm^{-1}. In our present study, benzene ring overtones were not observed in the IR spectra of any of the species (Fig. 1A–H). This could indicate that benzene-containing amino acids such as phenylalanine and tyrosine are absent in all of the species in this study. Distinct benzene ring overtones were only seen in three of the six terrestrial slugs, in only one of the two terrestrial gastropods, and totally absent in the aquatic gastropod studied by Skingsley *et al.* (2000). Thus, there is no consistent association between the presence or absence of benzene ring overtones with species, genera or habitat modes of gastropods.

The IR spectra of solids and liquids, in the present study, are expected to show broad absorption bands which tend to overlap. However, the overlapping may not seriously affect the peak positions. It may provide a spectral morphology which is unique to each species. However, due to presence of water inherent in the mucus, spectral features may be altered significantly. Therefore, it is not reliable to use spectral morphology as a tool to differentiate species.

We conclude that it is premature to advocate that IR spectroscopy could be used as a tool for species identification as suggested by Skingsley *et al.* (2000) owing to the many inconsistencies present in all the species studied to-date. However, from our results, it appears that band area ratios of the amine peaks may represent a potentially useful tool in distinguishing species.

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